C-terminal agrin fragment - a new fast biomarker for kidney function in renal transplant recipients

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Abstract: Background: The C-terminal agrin fragment (CAF) is a cleavage product of agrin, the major proteoglycan of the glomerular basement membrane. This article studies if CAF could serve as a biomarker for renal function in renal transplant recipients. Material and Methods: We measured serum CAF and creatinine concentrations and calculated estimated glomerular filtration rate (eGFR) (MDRD) in 96 healthy individuals and in 110 end-stage renal disease patients undergoing kidney transplantation before and after transplantation. Correlation between CAF and creatinine concentrations/eGFR was calculated as within-patient (cWP) and between-patient correlations (cBP). Moreover, we evaluated the association of CAF with delayed graft function (DGF). The diagnostic value of CAF for early detection of DGF compared to creatinine was evaluated by receiver operating characteristics (ROC) analysis. Results: CAF concentrations strongly correlated with creatinine (r = 0.86 (cWP), r = 0.74 (cBP)) and eGFR (MDRD) (r = 0.86 (cWP), r = 0.77 (cBP)). Pre-transplant (pre-Tx) CAF concentrations were 19-fold higher than in healthy individuals (1,115.0 (258.4-3,990.0) vs. 56.6 (20.0-109.5) pM). After transplantation, CAF decreased significantly faster than creatinine (postoperative days 1-3 (POD 1-3): 562.8 (101.6-2,113.0) pM; creatinine: pre-Tx 6.9 (3.1-15.7), POD 1-3: 6.4 (1.7-12.7) mg/dl, $p < 0.001$). Stable concentrations were reached 1-3 months after transplantation for CAF and creatinine (CAF 145.1 (6.7-851.0) pM; creatinine 1.6 (0.7-8.0) mg/dl). CAF concentrations at POD 1-3 were significantly associated with DGF and outperformed creatinine in early detection of DGF (area under the curve (AUC) CAF 80.7% (95% CI 72.3-89.1%) vs. AUC creatinine 71.3% (95% CI 61.8-81.1%), p = 0.061). Conclusion: CAF is a promising new and fast biomarker for kidney function and may serve as a new tool for the early detection of DGF.

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C-terminal agrin fragment (CAF) –

a new biomarker for evaluating kidney function

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Abstract

Background: C-terminal agrin fragment (CAF) is a split product of agrin, the major proteoglycan of the glomerular base membrane. The involvement of CAF in predicting glomerular filtration rate (eGFR) has never been evaluated.

Material&Methods: We measured/calculated serum CAF-, creatinine-levels and eGFR(MDRD) in 96 healthy individuals and in 110 end-stage renal disease (ESRD) patients undergoing kidney transplantation before and numerous times after transplantation. Correlation between CAF- and creatinine-levels/eGFR was calculated as within-patients- (cWP) and between-patients-correlation (cBP). Moreover we evaluated the association of CAF with delayed graft function (DGF). CAF's predictive value was compared to creatinine by receiver operating characteristics (ROC)-analysis.

Results: CAF levels strongly correlated with creatinine (r=0.86(cWP), r=0.74(cBP)) and eGFR(MDRD) (r=0.86(cWP), r=0.77(cBP)). Pretransplant (pre-Tx) CAF-levels were 19-times higher than in healthy individuals (1075.0 (112.0;6397.4) vs. 56.6 (20.0;109.5) pM). After transplantation, CAF levels decreased significantly faster than creatinine levels (postoperative day 1-3 (POD 1-3): 578.0 (101.6;2113.0), 54% of pre-Tx levels, creatinine: pre-Tx 6.8 (2.3;15.7) mg/dl, POD 1-3: 6.3 (1.1;12.7), 93% of pre-Tx levels, $p<0.001$). Stable serum levels were reached 1-3 months after transplantation in CAF and creatinine (CAF: 139.4 (6.7;851.0) pM, 13% of pre-Tx levels; creatinine: 1.6 (0.7;8.8) mg/dl, 24% of pre-Tx levels). CAF-levels at POD 1-3 were significantly associated with DGF and outperformed creatinine in predicting DGF (area under the curve(AUC)-CAF: 80.7% (95%-CI: 72.3%-89.1%) vs. AUC-Creatinine: 71.3% (95%-CI: 61.8%-81.1%, p=0.061)).

Conclusion: CAF is a new promising biomarker for kidney function. Moreover, it may serve as a new tool for the prediction and early detection of DGF.

Introduction:

Serum creatinine and urea are the most reliable biomarkers to monitor kidney function (1, 2). Although they have been used over decades and are applied for the evaluation of kidney function in the majority of studies, their application is limited: they lack sensitivity and specifity, especially in acute changes of kidney function and are influenced by multiple parameters such as muscle mass, liver function and pharmacological substances (1, 2). Thus, new biomarkers have been evaluated, for example cystatin c, human neutrophil gelatinase-associated lipocalin (NGAL), Interleukin-18 (IL-18) and Kidney-injury molecule 1 (KIM-1) (3, 4, 5, 6). But so far only cystatin c measurements have in part been routinely established.

Neurotrypsin, a serine protease, cleaves agrin, a major heparan sulfate proteoglycan, at two homologous sites, liberating a 22-k-Dalton sized C-terminal fragment, called "CAF" (7, 8, 9, see suppl 1). Among neuronal and other tissues, agrin is expressed in the kidney, where it substantially contributes to the formation of the glomerular basement membrane (GBM) (10, 11, 12), possibly linking it to the podocytes. CAF is detectable in human blood. The question rises, if changes of kidney function cause changes in CAF serum levels in human. So far CAF has never been scientifically explored in nephrological interrogations.

This is the first study in that field ever to characterize CAF as a biomarker for kidney function in healthy subjects as well as in patients with chronic renal failure. Thus, we evaluated serum CAF levels in healthy subjects and in 110 renal transplant recipients before and several times after transplantation to correlate CAF levels with kidney function. Additionally we addressed the question if early postoperative CAF levels could predict a delay of graft function (DGF) in the short term after transplantation.

Patients and Methods

Study population

The study was approved by the local ethic's committee. All patients enrolled in this study gave their consent. The total study population consisted of 206 individuals and was based on an observational study concept. Two different groups of patients were included in this study: 96 healthy volunteers and 110 patients suffering from chronic kidney disease (CKD) undergoing kidney transplantation.

Kidney transplant recipients

To evaluate the influence of kidney function on CAF levels we included 110 patients suffering from CKD or end-stage renal disease (ESRD). Patients underwent kidney (living as well as cadaveric donors) or combined kidney-pancreas-transplantation in the time from 2007 to 2011 at Klinikum rechts der Isar, Munich, Germany. Not every patient who received a kidney transplant from 2007 to 2011 was enrolled in this study. No specific inclusion or exclusion criteria had to be met. All patients received an initial triple immunosuppression consisting of a calcineurin-inhibitor (71 tacrolimus (TAC), 39 cyclosporine (CyA)), mycophenolic acid (MMF) and corticosteroids (Tab. 1). In the follow up period 16 patients were switched from CyA to TAC, whereas four patients were switched from TAC to CyA. Blood samples were obtained right before transplantation and several times up to a median of 128 days after transplantation (range 6-1757 days). In total 746 samples were obtained. The time points when blood was drawn did not follow a strict protocol, but blood samples could be obtained from every patient before and at least once in the early postoperative period (postoperative day 1-3, "POD 1-3"). Blood samples were categorized into certain time frames after transplantation (Tab. 1). If more than one

sample from one patient was obtained in a certain time frame, the mean CAF level was calculated and used for statistical work-up.

All blood samples were analysed for CAF and creatinine levels and estimated Glomerular filtration rate (eGFR). The eGFR was calculated for every sample using the formula of the MDRD-Trial (13). To analyse patient related parameters that might influence CAF levels, age, gender, weight and liver function parameters were assessed. At last we assessed the incidence of DGF, defined as the need for at least one dialysis treatment within the first week after transplantation (14). The need for dialysis treatment was evaluated by the treating physician and did not follow a strict protocol.

Healthy volunteers

The control group consisted of 96 healthy volunteers. Blood samples were drawn once in the morning hours to measure CAF and creatinine levels.

Blood sample measurement of CAF and creatinine levels

All blood samples were evaluated for CAF levels using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (NTCAF Elisa Kit, Neurotune, Schlieren, Switzerland, (15), see a detailed description there). In brief, 50 µl of blood sample need to be mixed with 50 µl of incubation buffer in wells on a Deepwell protein Lobind plate. After that 100 µl of 400 nM CAF Calibrator protein solution is mixed with 900 µl dilution buffer in order to create a calibrator dilution series on the same plate. Then the plate is sealed and incubated in a water bath preheated to 56°C ± 1°C for 30 min ± 1 min. When finished the plate is centrifuged for 5 min at 3000 x g at room temperature. Subsequently 10 µl of sample and dilution series is transferred

to a pre-coated micro titer plate, which is already prepared with 90 µl of dilution buffer in each well. Now the ELISA plate is incubated for 16 hours at room temperature. On the next day an ELISA analysis protocol has to be followed: the plate needs to be washed three times, after that 100 µl of CAF detector antibody solution is added to each well and incubated for 30 minutes at room temperature. The same step is repeated with SA-poly-HRP solution, followed by TMB solution for colour development. The results are read out in a plate reader at 450 nm. Data is analysed with an excel file supplied by the company. For every sample two ELISAs have to be performed and a mean value needs to be calculated. In the following text CAF values are expressed as picomol (pM), which corresponds to a concentration of 20 pg/ml.

Serum creatinine levels were quantified using a well established photometric measurement (Jaffe method, normal range 0.7-1.3 mg/dl in males and 0.5-1.1 mg/dl in females).

Statistics

For statistical analysis Excel (Microsoft, Redmond, USA) and SPSS software (SPSS Inc., Chicago, USA) were used. The Kolmogorov-Smirnov-Test was performed to evaluate the normality of data distribution. Continuous data are expressed as mean with standard deviation (SD) or median and range whenever appropriate. Categorial variables are reported in absolute numbers and percentages. To assess correlation between CAF and creatinine levels/eGFR a within-patient-correlation (cWP, in case of at least three samples obtained in one patient) as well as a between-patient-correlation (cBP) was calculated by using multiple regression (16, 17). Since we evaluate several samples in each individual, it can be misleading to analyse the whole set of samples as if the data were a simple set of samples. Therefore we tried to

answer two questions: we used the cWP to analyse if changes of CAF levels in one patient are associated with changes of creatinine levels in order to remove differences between patients. To answer the question if patients with high CAF levels also tend to have high levels of creatinine we used the cBP. Because the relationship between CAF and creatinine as well as eGFR can be linearized by a logarhythmic function, we also calculated correlations between logarhythmic CAF and logarhythmic creatinine/eGFR, according to the formula $y = a * xb$ that results in a linear expression $a \log(y) = a + b * \log(x)$ and tested this correlation for significance. To test if there is a kinetic difference in the decrease of post-operative CAF and creatinine levels compared to pretransplant (pre-Tx) levels Wilcoxon-Signed-Rank-Test test was used. Univariate analysis (using Spearman-rho test) was performed with CAF levels as dependent variable and the following covariables to evaluate if the following parameters influence pre-Tx CAF levels: age, gender, weight, alanin-aminotransferase (ALAT) and gamma-glutamyl-transferase (GGT). To analyse if there is an association of CAF and creatinine levels on POD 1-3 with DGF the Mann-Whitney test was performed. Additionally a receiver operating characteristic (ROC) analysis was performed to evaluate if CAF is an appropriate marker to predict DGF with accurate sensitivity and specifity and compared it to creatinine. A probability (p)<5% was considered statistically significant.

Results

Patients' demographics

The mean age of kidney transplant recipients was 51.2±13.5 years, 71 patients were male (Tab. 1). 103 patients received single kidney transplantation, in 79 patients a

deceased donor organ was transplanted. 40 (36.4%) patients experienced DGF. The mean age of healthy volunteers was 47.7±16.0 years, 34 were males (Tab. 1).

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Correlation of CAF and creatinine/eGFR in renal transplant patients

To assess the correlation of CAF and creatinine levels before and after transplantation we calculated the cWP and cBP as described in the statistics part. The cWP was r=0.68 ($p<0.001$, Tab. 2, Fig. 1a). When we calculated the correlation logarhythmic CAF and creatinine it was even stronger (r=0.86, $p<0.001$, Tab. 2, Fig. 1b). The cBP was somewhat weaker, but still r=0.55 ($p<0.001$, Tab. 2) for absolute values and r=0.74 ($p<0.001$, Tab. 2) for logarhythmic data.

When we compared logarhythmic CAF and eGFR the cWP was r=-0.86 ($p<0.001$, Tab. 2). The cBP was r=-0.77 ($p<0.001$, Tab. 2, Fig. 2). We did not calculate the correlation of absolute values, since the calculation of eGFR from creatinine values follows a logarhythmic approach.

Development of CAF levels and creatinine levels/eGFR in kidney transplant recipients after transplantation

Median pre-Tx CAF levels were 19 times higher than in healthy volunteers (1075.0 (112.0;6397.4) pM vs. 56.6 (20.0;109.5) pM, Tab. 2). Median creatinine levels in healthy subjects were 0.78 mg/dl (0.53;1.08). On POD 1-3 there was a 46% decrease in median CAF levels (578.0 (101.6;2113.0) pM, $p<0.001$, Tab. 2, Fig. 3) compared to pre-Tx levels. 1-3 months after transplantation CAF levels were around 87% lower than pre-Tx levels and 2.5 times higher than in healthy volunteers (139.4 (6.7;851.0), $p<0.001$, Tab.2, Fig. 3a/b). Creatinine levels decreased only by around 7% from 6.8 (2.3;15.7) mg/dl pre-Tx to 6.3 (1.1;12.7) mg/dl on POD 1-3. 1-3 months after

transplantation creatinine levels were 76% lower compared to pre-Tx levels (1.6 (0.7;8.8) mg/dl, p<0.001, Tab. 2, Fig. 3a/b) and 2.1 times higher than in healthy subjects (p<0.001).

Comparison of the development of postoperative CAF and creatinine levels within patients

Comparing the time course of both parameters in each patient, median CAF levels decreased significantly faster from pre-Tx to POD 1-3 than creatinine levels (46% vs. 7%, p<0.001, Fig. 3b). CAF levels again decreased more than creatinine levels from POD 1-3 to day 4-10 (53% vs. 38%, p=0.05, Tab. 2, Fig. 3b). From day 4-10 to day 11-30 the decrease of creatinine was greater (37.1% vs. 48.7%, p>0.05, Tab. 2, Fig. 3b). From day 11-30 to 31-90 the decrease of both parameters was not significantly different any more (CAF: 20% vs. creatinine: 18.4%, p>0.05, Tab. 2, Fig. 3b). After that time the development was statistically similar, since at that time a stable level on both parameters was reached. When we compared creatinine and CAF levels of each time period to pre-Tx levels, CAF levels were significantly lower at each time period compared to creatininie levels (p<0.01 for each time period, Fig. 3b).

CAF levels and DGF

40 (36.4%) patients experienced DGF. When we compared the correlation of pre-Tx CAF and creatinine levels with DGF, there was no statistical significant difference between both groups (p=0.688 and p=0.828, respectively, Tab. 3). On POD 1-3, CAF and creatinine levels were significantly higher in the DGF-group (p<0.001 and p=0.001, respectively, Tab. 3): In the DGF-group, CAF levels decreased around 19.9%, whereas creatinine levels increased by around 4.4% (Tab. 3). In the group

without DGF, CAF levels decreased around 64.6%, whereas creatinine levels decreased around 18.9%. In ROC-Analysis CAF levels on day 1-3 were moderately accurate in predicting DGF concerning sensitivity and specifity with an area under the curve (AUC) of 80.7% (72.3%-89.1%, Fig. 4). It was superior to creatinine with an AUC of 71.3% (61.8%-81.1%, p=0.061, Fig. 4). The optimal cut-off value for CAF in predicting DGF was 676.5 pM, resulting in a sensitivity of 74.4% and a specifity of 81.2% (Tab. 4). The optimal cut-off for creatinine was 5.4 mg/dl, resulting in a sensitivity of 92.3%, but a specifity of only 50.0% (Tab. 4).

Effect of different parameters on CAF and creatinine levels

On univariate analysis, median pre-Tx CAF levels in females were significantly higher than in males (1206 (112.0;3990.0) pM vs. 983 (259.0;6397.0) pM, p=0.032). Age (p=0.985), weight (p=0.547) and liver function (ALAT p=0.547, GGT p=0.461) had no major impact on CAF levels.

Discussion

This is the first study ever demonstrating that CAF could serve as a new and reliable biomarker for evaluation and monitoring of kidney function.

In detail we could firstly show that CAF levels highly correlated to eGFR as well as creatinine levels. CAF levels were 19x higher in ESRD patients before transplantation compared to healthy subjects. Secondly, CAF levels decreased significantly over a period of 4 weeks to reach stable levels at 1-3 months after transplantation. The decrease exceeded and was faster than that of creatinine. Thirdly, stable CAF levels were lower than creatinine levels compared to pre-Tx levels, indicating a wider range of data and therefore higher sensitivity for small changes of kidney function.

Fourthly, early postoperative CAF levels were statistically significantly associated with DGF and even more predicted DGF with good sensitivity and specifity exceeding the values of creatinine.

The major finding of our study is that CAF levels highly correlated to eGFR and creatinine levels. Basically there are two possible explanations for this finding: CAF is a cleavage product of agrin, mediated by neurotrypsin, a serine protease. Agrin itself is a major component of the synapse and different base membranes, among them the GBM (7, 8, 9). Increased CAF levels could be generated either due to reduced glomerular filtration or tubular secretion (which is conceivable and also possible because of the size of 22 k-Da). This would be analogous to creatinine and is likely due to the similar kinetics both biomarkers shown with improving kidney function (Fig. 3a/b). Otherwise the cleavage of agrin directly in renal tissue could possibly lead to a degradation of the GBM and therefore of the glomerulus itself, causing a decline in glomerular function and/or glomerular loss respectively. This may as a consequence lead to elevated CAF levels.

The second finding of our study was the observation of a decrease in CAF blood levels with improving graft function after transplantation. In statistical analysis a strong correlation of CAF and creatinine levels/eGFR was detected in transplant recipients for both the absolute levels and the relative changes after transplantation in single patients. In this context the correlation of changes in both parameters exceeded the absolute values correlation, indicating a strong dependence of CAF on renal function since absolute creatinine values show high intraindividual differences and therefore correlations might be biased when comparing absolute values. Interestingly the logarhythmic correlation between CAF and creatinine as well as eGFR was higher than comparing absolute values. For eGFR, which is being calculated from creatinine

blood levels in a logarhythmic approach, this hyperbolic correlation was also observed for other biomarkers such a cystatin c, NGAL and creatinine (18, 19, 20, 21, 22), but correlation of CAF and eGFR in the setting of transplantation was even stronger than shown for cystatin c and NGAL (21, 23, 24, 25). Compared to creatinine it is remarkable that in the setting of severely reduced renal function (GFR <20 ml/min) the percentual change of CAF values with each ml/min of eGFR is much higher than in creatinine, ranging from around 200 to 2000 pM, compared to creatinine with a smaller variation in this stage of kidney disease. Compared to pre-Tx levels, CAF levels were significantly lower in every time period after transplantation compared to creatinine. Overall, the range of CAF levels in our study was from 139.4 pM (median) at stable graft function 1-3 months post-transplant to 1075 pM (median) pre-transplant, indicating a variation of nearly 800%, whereas the range of creatinine was only half as much at around 400%. This suggests that CAF is a more sensitive and faster marker for detection of also smaller changes of renal function, a finding that could be of outstanding value. This observation is fortified by the finding that CAF levels decreased significantly faster than creatinine in the early postoperative phase, whereas creatinine showed a more slothful reaction on improvement of transplant function. This results in both markers reaching stable blood levels 1-3 months after transplantation. Similar observations have been published for cycstatin c and NGAL in the setting of kidney transplantation (20, 24, 26, 27) being superior to creatinine in early reaction of blood levels on changes of kidney function. But other studies failed to show superiority of cystatin c and its calculation formulas as well as NGAL over creatinine in reflecting renal function in different settings (28, 29, 30, 31). Additionally cystatin c levels were observed to rise again in the postoperative

period after renal transplantation independently from renal function (26, 32), therefore limiting its use under these clinical circumstances.

The third major finding is a significant association of CAF levels on POD 1-3 with DGF. CAF levels on day 1-3 after transplantation predicted DGF with higher specifity and sensitivity than creatinine. DGF is a major issue in the early postoperative phase, since adverse events such as immunologic hazards need to be handled quickly. Therefore a sensitive marker is urgently needed. In our collective 36.4% of patients experienced DGF. In this group elevated CAF levels on POD 1-3 were significantly associated with DGF with being more than twice as high as in the group without DGF, whereas creatinine levels only differed by around 20%. Reversely, CAF levels decreased by around 63% in the group without DGF, compared to 20% in the DGF group. In ROC-analysis, CAF levels on POD 1-3 predicted DGF moderately accurate with higher sensitivity and specifity than creatinine at an optimal cut-off value of 677 pM. Urine NGAL and IL-18 have been evaluated for the same purpose (33, 34, 35), often with good results. But serum markers are favoured since urine often is hard to assess. Whereas serum IL-18 failed to be of great value for the prediction of DGF in renal transplant patients when measured in the blood (36, 37), results on NGAL are conflicting (37, 38, 39, 40). Blood cystatin c levels were beneficial in some studies (37, 41, 42), but failed in other publications to correlate accurately with allograft function in the early postoperative phase (26, 32, 43). However, compared to other biomarkers CAF appears to be a promising marker in this setting.

When we evaluated parameters possibly influencing CAF levels we detected that in univariate analysis female sex had slightly higher CAF levels than males. Apart from that, neither weight nor age influenced CAF levels. Consindering the influence of gender on CAF levels, which is also known for creatinine and cystatin c (44), CAF

seems to be a robust parameter for kidney function independent from individual parameters.

Our study has limitations. It is only based on clinical observative data and does not deliver pathophysiological background like glomerular filtration and tubular secretion/absorption of CAF. Future studies will have to follow to characterize the role of neurotrypsin, CAF and its kinetics on a molecular and histological level. Additionally the CAF and creatinine levels assessed were in time frames but not specific postoperative days. This would have been desirable to characterize the blood level kinetics in a more detailed way.

Taken together, CAF might be potential new biomarker for kidney function with a high level of sensitivity and specifity that could possibily exceed the value of creatinine and cystatin c. Additionally CAF might serve as a good clinical biomarker to predict DGF accurately in kidney transplant recipients. Based on the results of our study, future clinical trials evaluating CAF in different clinical settings such as acute kidney failure or chronic diseases should be initiated.

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Table 1: Kidney allograft recipient's demographics

Parameter	Result
Age (years, mean +/- SD)	*51,2 (+/- 13,5)*
Gender (n, %)	*110 (100%)*
Male	71 (64.5%)
Female	39 (35.5%)
Transplantation (n, %)	*110 (100%)*
Kidney	103 (93.6%)
Kidney-pancreas	7 (6.4%)
Kind of donation (n, %)	*110 (100%)*
Deceased donor	79 (71.8%)
Living donor	31 (28.2%)
Underlying renal disease (n, %)	*110 (100%)*
Diabetic nephropathy	23 (20.1%)
Vascular nephropathy	12 (10.9%)
Autosomal polycystic kidney disease (ADPKD)	11 (10.0%)
Immunogenic (IgA-Nephropathy,GwP, LE, MPGN)	32 (29.1%)
Other (hereditary, intersitial disease, unknown)	32 (29.1%)
Immunosuppression – Calcineurininhibitor (n, %)	
Tacrolimus	67 (60.1%)
Cyclosporine	23 (20.1%)
Switch from Cyclosporine to Tacrolimus	16 (14.5%)
Switch from Tacrolimus to Cyclosporine	4 (3.6%)
Patients with delayed graft function (n, %))	*40 (36.4%)*
Samples obtained in total (n, %)	*746 (100%)*
Before transplantation	110 (14.7%)
1-3 days after transplantation	127 (17.0%)
4-10 days after transplantation	131 (17.6%)
11-30 days after transplantation	98 (13.1%)
30-89 days after transplantation	123 (16.5%)
90-179 days after transplantation	88 (11.8%)
6-12 months after transplantation	48 (6.4%)
>12 months after transplantation	21 (2.8%)

Data are presented as absolute numbers (n) and percentrage (%) in brackets; SD = stan-dard deviation; GwP = granulomatosis with polyangiitis; LE = Lupus erythematodes; MPGN = membranoproliferative Glomerulonephritis;

Table 2: CAF levels, creatinine levels and eGFR (MDRD) in renal transplant recipients before and after transplantation

Samples	CAF (pM)	creatinine (mg/dl)	cWP CAF-creatinine	cBP CAF-creatinine	eGFR (MDRD, ml/min)	cWP CAF-eGFR	cBP CAF-eGFR
Before Tx	1075.0 (112.0;6397.4)	6.8 (2.3;15.7)			8 (4;25)		
day 1-3	578.0 (101.6;2113.0)	6.3 (1.1;12.7)	absolute	absolute	9 (4;55)		
day 4-10	271.6 (57.1;1933.0)	3.9 (0.7;17.6)	0.68*	0.55*	17 (4;106)		
day 11-30	170.9 (46.2;1337.0)	2.0 (0.8;10.0)			36 (6;111)	logarhythmic	logarhythmic
day 31-90	139.4 (6.7;851.0)	1.6 (0.7;8.8)			43 (7;130)	-0.86*	-0.77*
day 91-180	151.3 (13.7;509.0)	1.8 (0.7;5.0)	logarhythmic	logarhythmic	40 (12;130)		
day 181-365	143.6 (11.3;483.1)	1.6 (0.7;3.7)	0.86*	0.74*	43 (14;129)		
more than 365 days	140.5 (46.3;287.6)	1.7 (0.9;2.5)			44 (22;78)		

Data are presented as median (minimum;maximum); cWP = within-patient-correlation; cBP = between-patient-correlation; CAF = C-terminal agrin fragment; eGFR = estimated glomerular filtration rate; pM = picomolar; mg/dl = miligrams/decilitre; ml/min = mililitre/minute; Tx = transplantation; *p<0.001;

Table 3: CAF and creatinine levels in patients with and without delayed graft function

	Patients with DGF	Patients w/o DGF	p value
n	40	70	
pre-Tx CAF levels (in pM)	1137 (366;2133)	1031 (258;6397)	0.688
POD 1-3 CAF levels (in pM)	911 (271.7;1763)	364.6 (102;2113)	<0.001
pre-Tx creatinine levels (in mg/dl)	6.8 (3.7;15.7)	6.9 (3.1;14.2)	0.828
POD 1-3 creatinine levels (in mg/dl)	7.1 (2.9;12.7)	5.6 (1.7;12.5)	0.001

Data are presented in median (minimum;maximum); DGF = delayed graft function; w/o = without; CAF = C-terminal agrin fragment; n = number of patients; pre-Tx = pre-transplant; POD = postoperative day; pM = picomolar; mg/dl = miligrams/decilitre;

Table 4: Sensitivity and specificity of different CAF and creatinine cut-off values on POD 1-3 for predicting DGF

Biomarker	Cut-off level	Sensitivity	Specify
CAF (in pM)	1304.5	15.4%	95.7%
	676.5	74.4%	81.2%
	283.5	97.4%	37.7%
Creatinine (in mg/dl)	9.6	15.4%	95.7%
	5.4	92.3%	50.0%
	3.5	97.4%	12.9%

CAF = C-terminal agrin fragment; POD = postoperative day; DGF = delayed graft function; pM = picomolar; mg/dl = miligrams/decilitre;

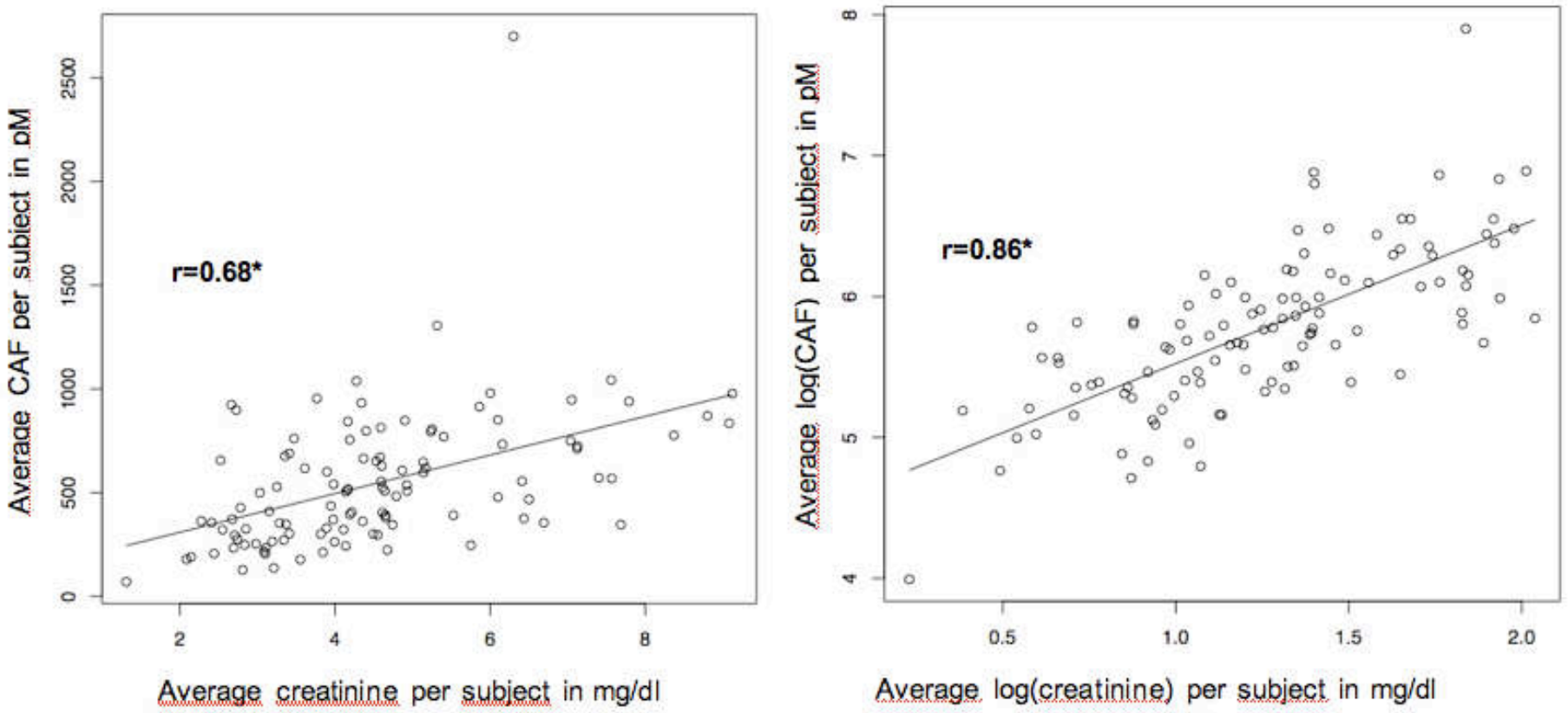

Figure 1. a: within-patient-correlation between CAF and creatinine, absolute values; b: within-patient-correlation between CAF and creatinine, logarhythmic values;
CAF = C-terminal agrin fragment; pM = picomolar; mg/dl = miligrams/decilitre; log = logarhythmic; r = correlation; *p<0.001

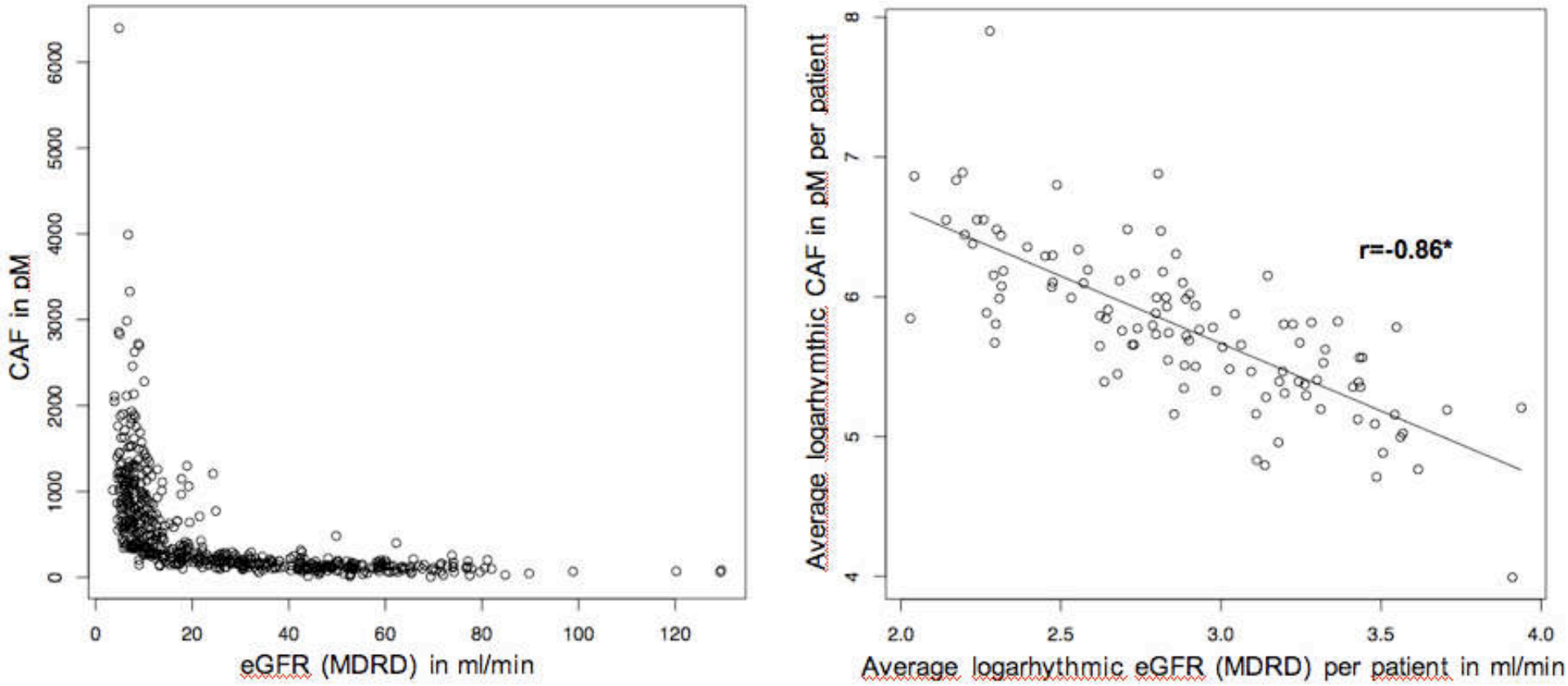

Figure 2. a: comparison of corresponding absolute values of CAF and eGFR for every sample; b: between-patient-correlation for logarhythmic CAF and eGFR;
CAF = C-terminal agrin fragment; pM = picomolar; eGFR = estimated glomerular filtration rate; ml/min = mililitre/minute; r = correlation;
*p<0.001

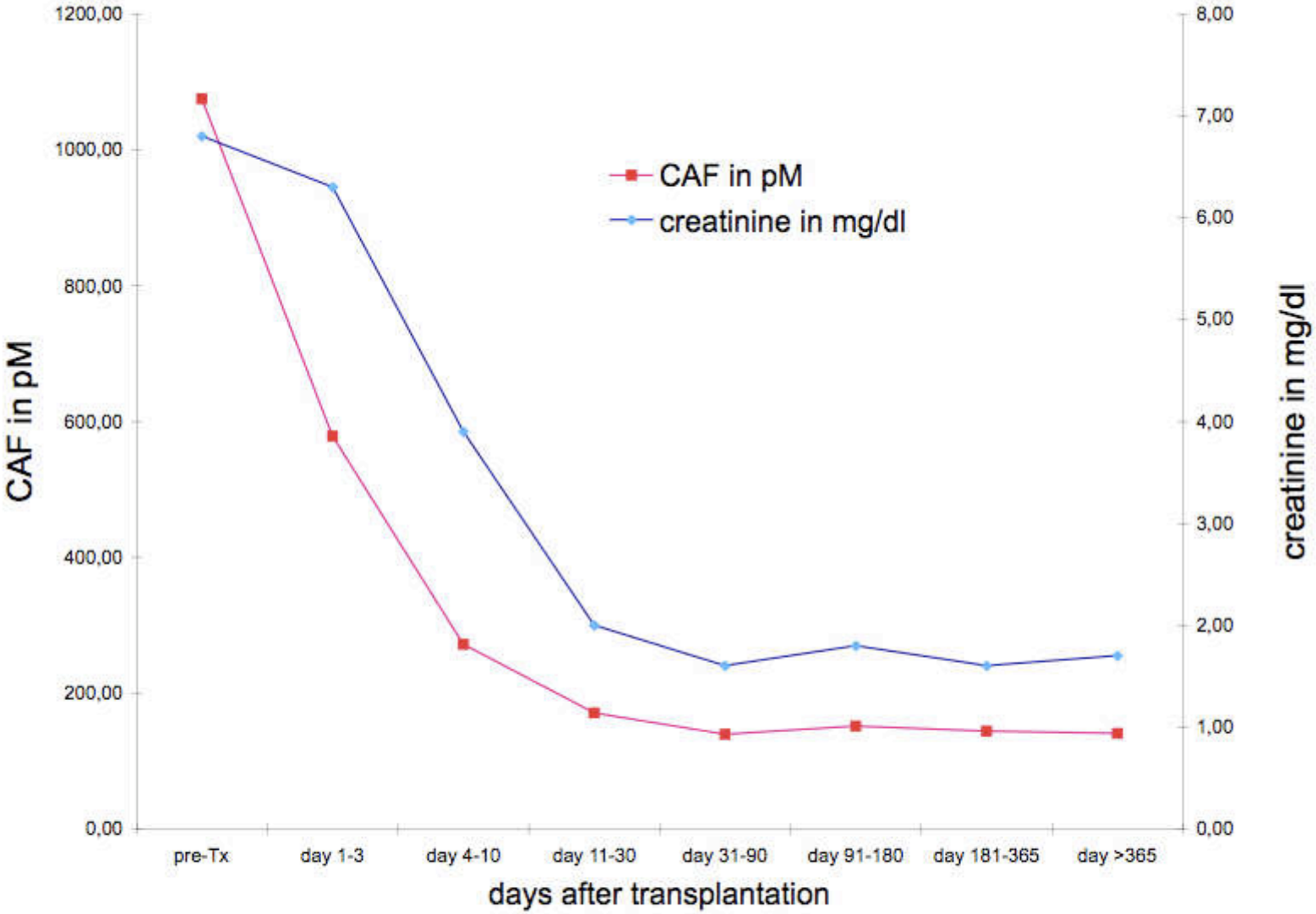

Figure 3a: development CAF and creatinine levels in renal transplant recipients before and after transplantation;
values are expressed in mean; CAF = C-terminal agrin fragment; pM = picomolar; pre-Tx = pre-transplantation; mg/dl = miligrams/decilitre;

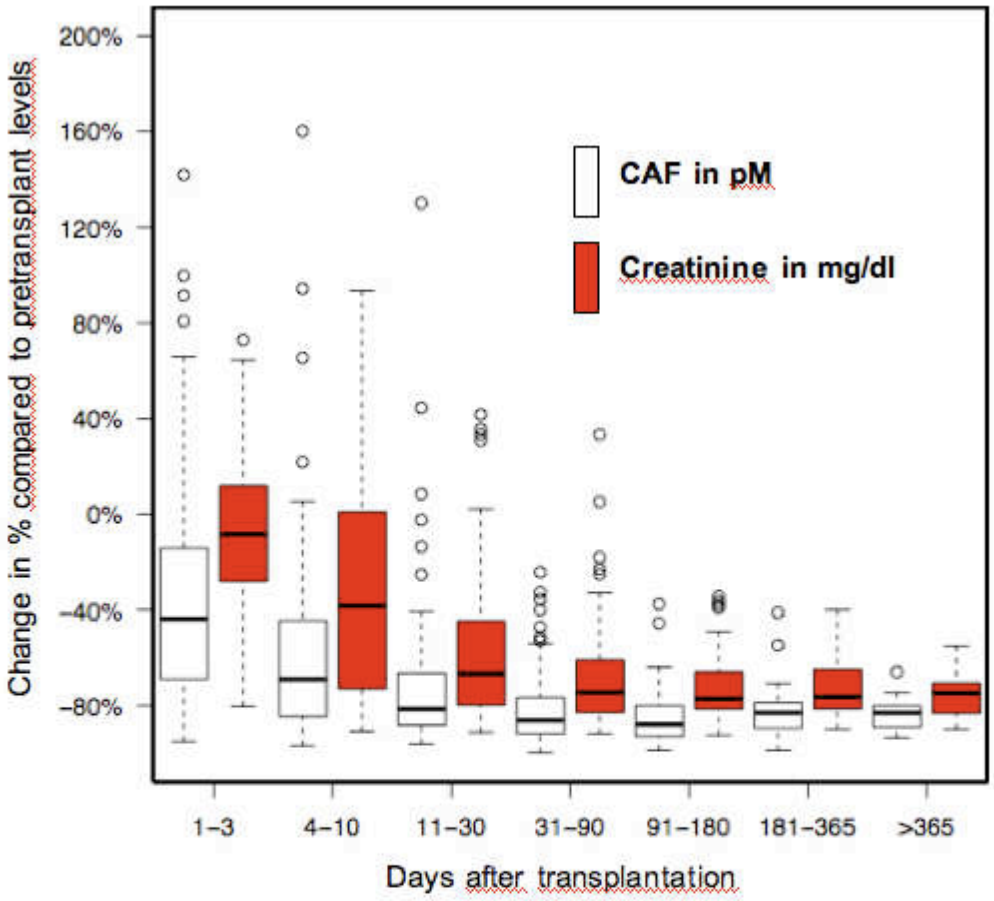

Fig. 3b: change of CAF/creatinine levels at each time period in reference to pretransplant levels;
Results presented as boxplots: horizontal black line indicates the median, box indicates 50% of all samples, dotted lines indicate 95% of all samples; circles indicate extreme values;
CAF = C-terminal agrin fragment; pM = picomolar; mg/dl = milgrams/decilitre;

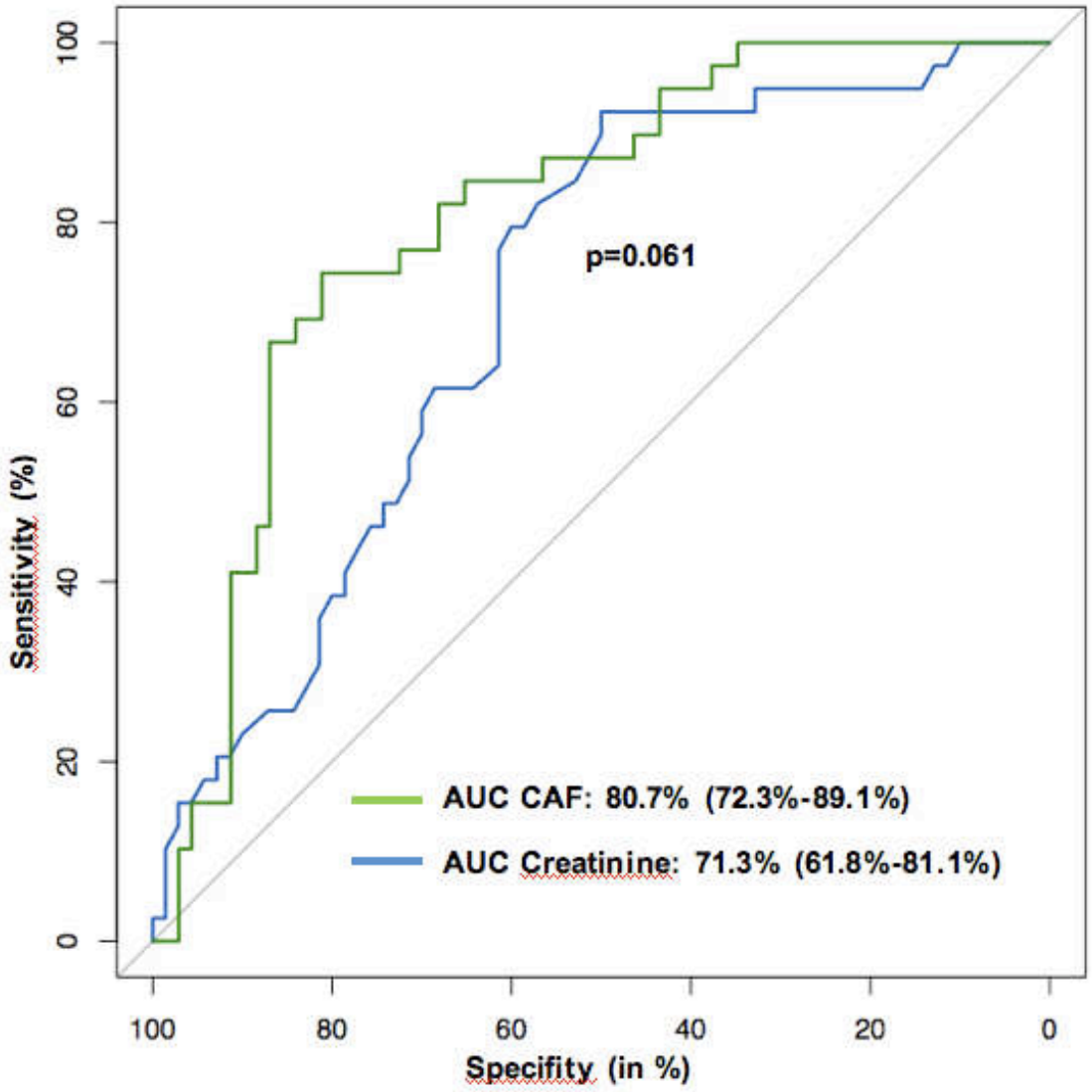

Figure 4. Receiver-operating-characteristics-analysis comparing CAF and creatinine on POD 1-3 in predicting delayed graft function;
AUC = area under the curve; CAF = C-terminal agrin fragment; POD = post operative day;